AD_____

Award Number: DAMD17-00-1-0359

TITLE: Dissecting Immunogenicity of Monoclonal Antibodies

PRINCIPAL INVESTIGATOR: Christopher M. Snyder
Lawrence J. Wysocki

CONTRACTING ORGANIZATION: The National Jewish Medical and Research Center
Denver, Colorado 80206

REPORT DATE: June 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual Summary (1 Jun 2000 - 31 May 2003)

4. TITLE AND SUBTITLE
Dissecting Immunogenicity of Monoclonal Antibodies

5. FUNDING NUMBERS
DAMD17-00-1-0359

6. AUTHOR(S)
Christopher M. Snyder
Lawrence J. Wysocki

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The National Jewish Medical and Research Center
Denver, Colorado 80206

E-Mail: christopher.snyder@uchsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The potential of mononclonal antibodies, (mAbs), for use in therapeutic and diagnostic applications has not been fully realized in part due to counter-immune responses that often arise in patient recipients of mAb. A growing research effort to "humanize" mAb has focused primarily on the structure or sequence of the antibody variable (V) region domains. However, these approaches may ultimately suffer, as they overlook the requirement of T cell help for the immune counter-reaction and the potential of somatic hypermutation and V-D-J recombination to generate target T cell epitopes within mAb V regions. My approach focuses on this issue. In order to understand some basic principals concerning anti-immunoglobulin immune responses, I have devloped a panal of T cell hybridomas, new transgenic mice and a tetrameric staining reagent. Studies with these tools strongly suppport our basic hypothesis that T cells are tolerant of endogenous immunoglobulin-derived diversity. I have also obtained a panal of T cell hybriomas that are specific for the CDR3 region of a monoclonal antibody supporting our hypthesis that junctional diversity may provide a source of T cell epitopes within a monoclonal antibody. Finally, I have addressed the global nature of T cell responses to junctional diversity with an adoptove transfer system.

14. SUBJECT TERMS
Tumor Therapy and Detection, Immunology Monoclonal Antibodies, Counter-Immune Response

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

Monoclonal antibodies have been increasingly used for therapeutic and diagnostic applications. Their potential use in targeting tumor antigens, specifically those expressed by carcinomas of the breast, is just now beginning to be appreciated. Successful application of monoclonal antibodies has been constrained by counter-immune responses elicited in patient recipients. Counter-immune responses can limit the duration and number of times that a patient can receive the therapy and may result in potentially dangerous reactions. Efforts to avoid this response have mostly centered on a variety of strategies to "humanize" monoclonal antibodies. The rationale behind this approach is that "humanized" antibodies will have few or no epitopes that could be viewed as "foreign" by the recipient's immune system. However, mounting an immune response to a protein antigen generally requires both B and T lymphocytes, and a memory response always requires both cell types. In general, current efforts to humanize monoclonal antibodies have overlooked potential T cell epitopes present within the mAb variable region.

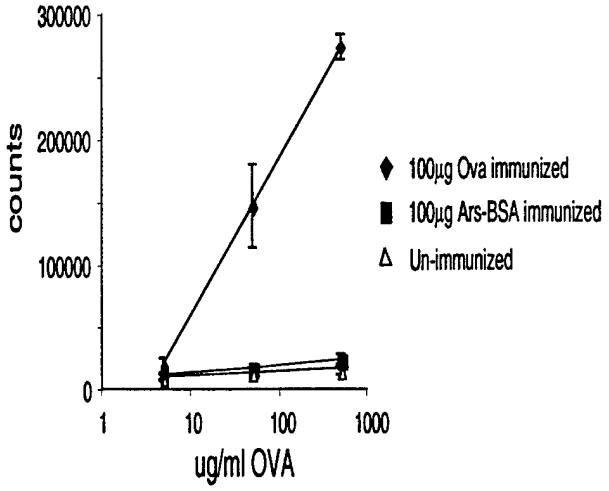
Previous data from several laboratories, including ours, have suggested that T cells can respond to epitopes created in immunoglobulin variable regions through the physiological somatic hypermutation process [1, 2]. Furthermore, it has been proposed that the generation of junctional diversity by V-D-J gene recombination can produce T cell epitopes. However, to my knowledge, this has not been extensively studied and incorporated as a humanization strategy. My view is that the oversight of these naturally occurring T cell epitopes is a major drawback to current humanization approaches. This proposal was originally aimed at understanding the prevalence of T cell epitopes in monoclonal antibodies. Through these analyses, we have assessed the nature of T cell responses to immunoglobulin V-region diversity present on soluble immunoglobulin and expressed by peripheral B cells.

Body:

In aim #1 of my proposal, I outlined a plan to assess the immunogenicity of an antibody with a germline-encoded T cell epitope in highly defined syngeneic mice. I proposed to immunize congenic strains of mice with a well defined germline-encoded T cell epitope located in the variable region of monoclonal antibody, (mAb), 36-65. The natural V-gene encoding this epitope has been bred onto the C58 background providing me with congenic C58 strains that either contain or lack the gene. These experiments aim to test the idea that germline encoded antibody sequences may be non-immunogenic while somatically generated epitopes are immunogenic even in syngeneic animals. Thus, if my hypothesis is correct, simply removing any somatically generated epitopes from a mAb may reduce its immunogenicity significantly.

I originally pursued the development of several techniques with which to analyze *ex vivo* immunoglobulin (Ig)-reactive T cells. First I chose to develop a lymph node proliferation assay sensitive enough to detect T cell responses to antibody variable regions. Initially, significant progress was made in measuring ex vivo, Ig-specific T cell proliferative responses (Figure 1). However, we found that the overall sensitivity of the assay was limited and often inconsistent. For these reasons, I have also pursued the analysis of T cell hybridomas derived from our congenic C58 strains. While arduous, this technique has been well worked out by our laboratory and has been successfully used for analyzing the T cell responses of C58 mice in the past [5]. For this analysis, either our C58 mice or our congenic C58 mice containing the germline VH gene used by mAb 36-65 are immunized with a peptide encoded by the germline gene in question. Lymph nodes from a recently, (3-4 days), boosted animal are then harvested and fused *in vitro*. Individual hybridomas resulting from the fusion are then screened for reactivity to both the immunizing peptide and the parent antibody. Over 300 T cell hybridomas were obtained from immunizations of these congenic strains of mice. The data are still being compiled, but to date confirm our previous findings indicating that C58 mice, which lack the variable gene, are indeed able to respond to epitopes encoded by this gene. Conversely, none of the tested hybridomas derived from congenic C58 mice that carried the 36-65 VH gene were found to have any immunoglobulin specific reactivity. The evidence obtained thus far confirms our hypothesis that the T cell repertoire is tolerant of germline-encoded isogenic antibodies. Thus somatically-generated diversity in the antibody V region is likely to be the only source of problematic T cell help against therapeutic monoclonal antibodies.

A:



B:

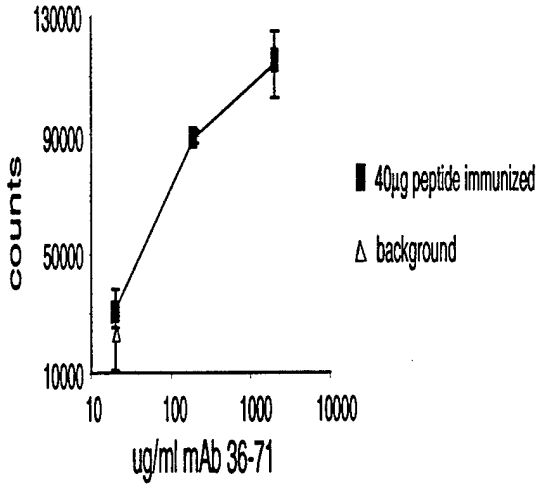
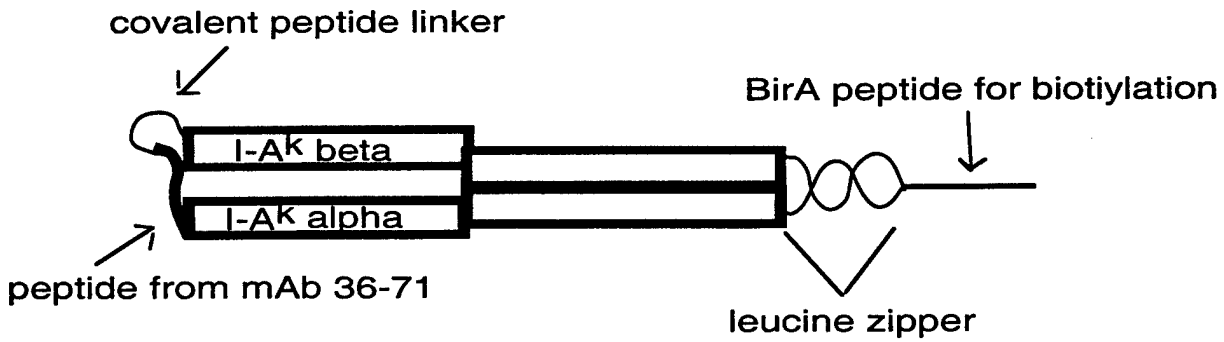


Figure 1: Examples of lymph node proliferation assays. The assay was performed by immunizing mice subcutaneously in the flank with the indicated amount of antigen. Draining inguinal and periaortic lymph nodes were harvested 14 days later from individual mice. T cells were purified from the lymph nodes using magnetic separation, (Stem Cell Technologies). 200,000 T cells and 400,000

splenocytes from an unimmunized mouse were plated with the indicated antigen concentrations. The background shown in figure B is derived from wells with no antigen added.

The proliferation assays and hybridoma sampling procedures were simply readouts that demonstrated T cell tolerance to germline Ig sequences. While the tolerance result was informative, the techniques used to demonstrate it were limited in that they did not yield information regarding the potential mechanisms by which tolerance was attained in T cells. In order to more closely analyze Ig-specific T cell tolerance both centrally (in the thymus) and peripherally, I devised an alternate strategy that involved visualizing T cells with class II MHC tetramers. When used in conjunction with T cell receptor (TCR) transgenic mice, the tetrameric staining reagents allowed me to visualize specific T cells participating in the immune response to immunoglobulin peptides, as well as T cells developing in the thymus. This approach was conducted concurrently with the techniques described above. Although this tetramer/TCR transgenic approach was not proposed in my original aims it, permitted me to address the tolerance question at a higher level of resolution than I originally proposed. Thus far, two such tetramers have been constructed. The first is an I-E^k-MHC construct containing the germline immunoglobulin epitope from mAb 36-65. Experiments are under way to verify its specificity. The second, is an I-A^k-MHC construct containing a mutant framework 1 (FR1) peptide peptide obtained from the Vκ-region of monoclonal antibody 36-71. Expression of the soluble I-A^k protein was challenging and ultimately required the addition of leucine zippers at the C-terminal end of both chains (diagramed in Figures 2A and B). This tetramer specifically stained T cell hybridomas that responded to the 36-71 VκFR-1 peptide in the context of I-A^k but not irrelevant T cell hybridomas (Figure 2C).

A:



B:

D I Q M T Q I P S S L S A----covalent peptide linker to I-A^k-beta

C:

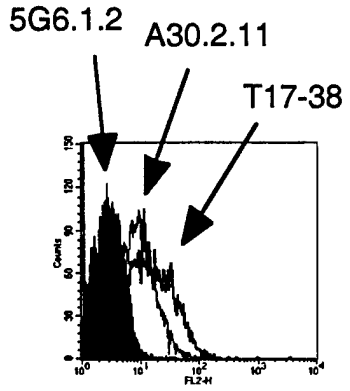


Figure 2: A) A schematic of the I-A^k tetrameric construct including the leucine zipper ultimately used for protein stability. B) The 13 amino acid peptide sequence derived from mAb 36-71 framework-1 region. The underlined isoleucine and proline are the amino

acids created by somatic hypermutation. C) FACS analysis of 3 different T cell hybrids stained with I-A^k 36-71. The two open histograms represent 36-71 specific hybridomas. 5G6.1.2 is an irrelevant hybridoma.

In addition to the I-A^k 36-71 MHC tetramers, I also successfully generated 36-71 specific TCR transgenic mice. These mice, which have been designated CA30 after the hybridoma from which the TCR genes were cloned, provide me with a monoclonal T cell population with which to study Ig-directed T cell responses. The T cells from the CA30 mice are specific for the VκFR1 peptide that is expressed in the I-A^k 36-71 MHC tetramers described above. The transgenic T cell receptor is expressed at a high level, as demonstrated by staining with the I-A^k 36-71 MHC tetramer (Figure 3A). Moreover, allelic exclusion appears to be intact as indicated by a virtual lack of endogenous alpha chain expression (Figure 3B). Finally, these T cells were highly functional as indicated by both proliferation and cytokine production (Figures 3C and 3D). The large number of immunoglobulin specific T cells in the TCR transgenic mice allows me to directly observe the development of tolerance to a variable region peptide.

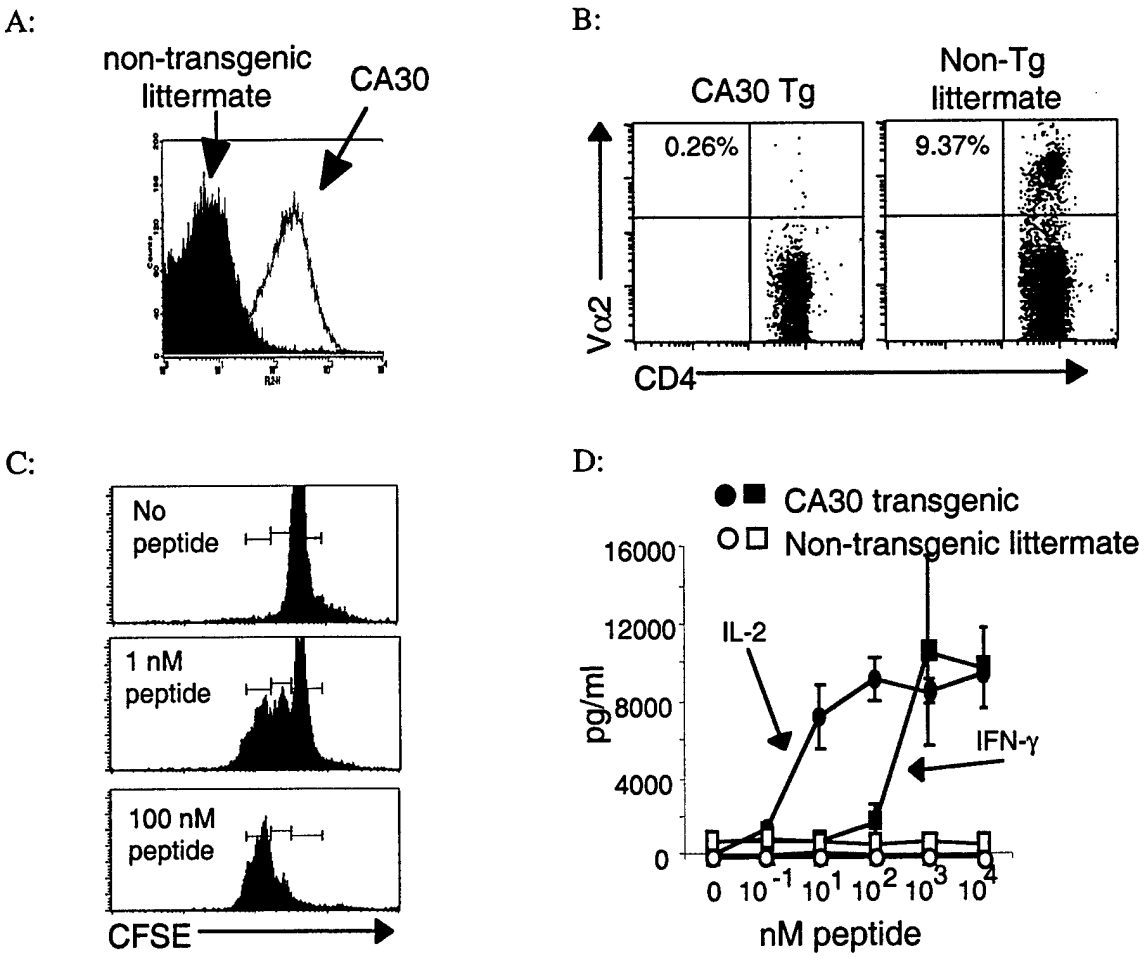


Figure 3: A) I-A^k 36-71 tetramer staining of peripheral lymph node CD4 cells from CA30 transgenic (green line) or non-transgenic littermate (filled histogram) mice. B) Expression of Vα2 (an endogenous TCR alpha chain) on peripheral lymph node CD4 cells from CA30 transgenic mice and non-transgenic littermates. C) CA30 lymph node cells were labeled with CFSE and stimulated with exogenous Vκ 36-71 FR-1 peptide Proliferating cells lose CFSE intensity over time. Figure C shows the FACS analysis of CFSE intensity for tetramer positive T cells after 2 days of stimulation with exogenous peptide. D) Concentration of IL-2 and IFN-γ secreted into the supernatant by 10^5 CA30 T cells after stimulation with exogenous Vκ 36-71 FR-1 peptide for 2 days.

Previous work in the laboratory led to the construction of an immunoglobulin transgenic mouse that expresses the mutated kappa-light chain from mAb 36-71. Thus, B cells from the Vκ 36-71 transgenic mouse express the epitope recognized specifically by the T cells from the A30 transgenic mouse. Together the tetrameric staining

reagent, the TCR transgenic mouse and the Vκ 36-71 transgenic mouse provided me with a more dynamic and physiological experimental model to address the specific question described in Aim #1.

In a first set of experiments, I generated mice expressing both the TCR and kappa-light chain transgenes, to investigate the phenotype of a T cell that is specific for an endogenous immunoglobulin variable-region peptide. Significant time and effort was expended in analyzing these mice. Strikingly, I observed a near complete deletion of Ig-specific T cells in the thymi of double transgenic offspring of CA30 transgenic and Vκ 36-71 transgenic mice. As shown in Figure 4A, virtually all (>97%) of the CD4+ T cells were deleted in the thymi in mice expressing both the T cells and the specific 36-71 epitope. This result was directly reflected by the lack of CD4 positive T cells in the lymph nodes of double transgenic offspring throughout the life of the animal (Figures 4A and B). These results were corroborated by tetramer staining of the thymi and lymph nodes of double transgenic offspring indicating that Ig-specific T cells were also lacking from the double transgenic mice (Figure 5 A and B). Moreover, T cells recovered from the lymph nodes of adult mice utterly failed to respond *in vitro* to the Vκ 36-71 FR1 peptide, confirming that the Ig-specific T cells were in fact deleted (Figure 5C).

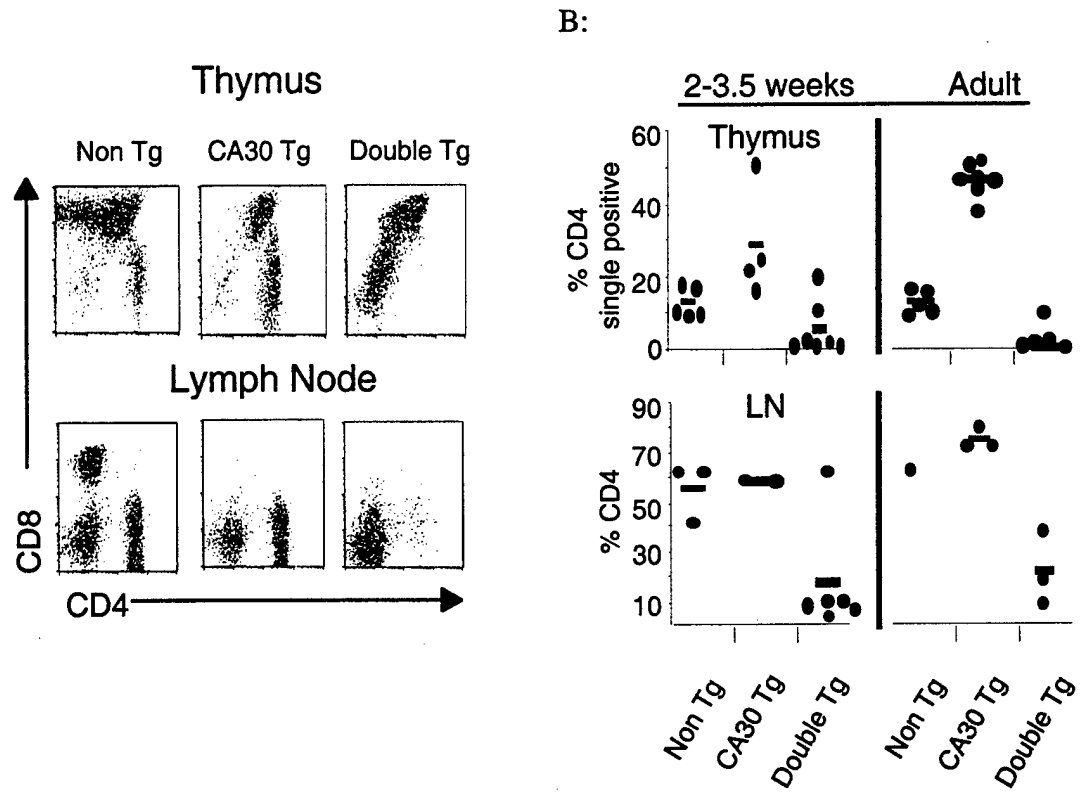


Figure 4. CA30 transgenic males were bred with Vκ 36-71 kappa transgenic females. A) Representative FACS plots of thymic and lymph node cells from double transgenic, CA30 single transgenic (derived from TCR only breeders), and Vκ36-71 single transgenic adult mice stained with CD4 and CD8. B) Frequencies of single positive CD4+ thymic T cells (upper panel) and lymph node CD4+ T cells (lower panel) from multiple mice represented graphically. Each circle represents an individual mouse.

This analysis demonstrated that Ig-specific T cells could be centrally deleted in the presence of Ig-bearing B cells and soluble antibody. To more closely analyze this phenomenon, I bred heterozygous CA30 transgenic males with heterozygous Vκ 36-71 females. Offspring of this cross that express only the CA30 TCR transgene are nonetheless exposed to Vκ 36-71 antibody in soluble form due to transfer through the placenta and in the mother's milk. Thus, I could assess the ability of soluble antibody alone to tolerize Ig-specific T cells. Indeed, early in the life of the offspring my results were identical to those obtained previously. CA30 transgenic T cells were centrally deleted by soluble antibody alone (Figure 6A). Strikingly however, the transgenic population recovered with time after weaning, presumably because of a gradual decline in the level of circulating Vκ 36-71

antibody (Figure 6B). Thus, Ig-specific T cells can be tolerized by soluble immunoglobulin, but tolerance only persists as long as the soluble antibody is present.

In sum, these data confirm our basic hypothesis that T cells are tolerant of endogenous germline-encoded peptides derived from immunoglobulin variable regions, and they reveal that tolerance may occur by clonal

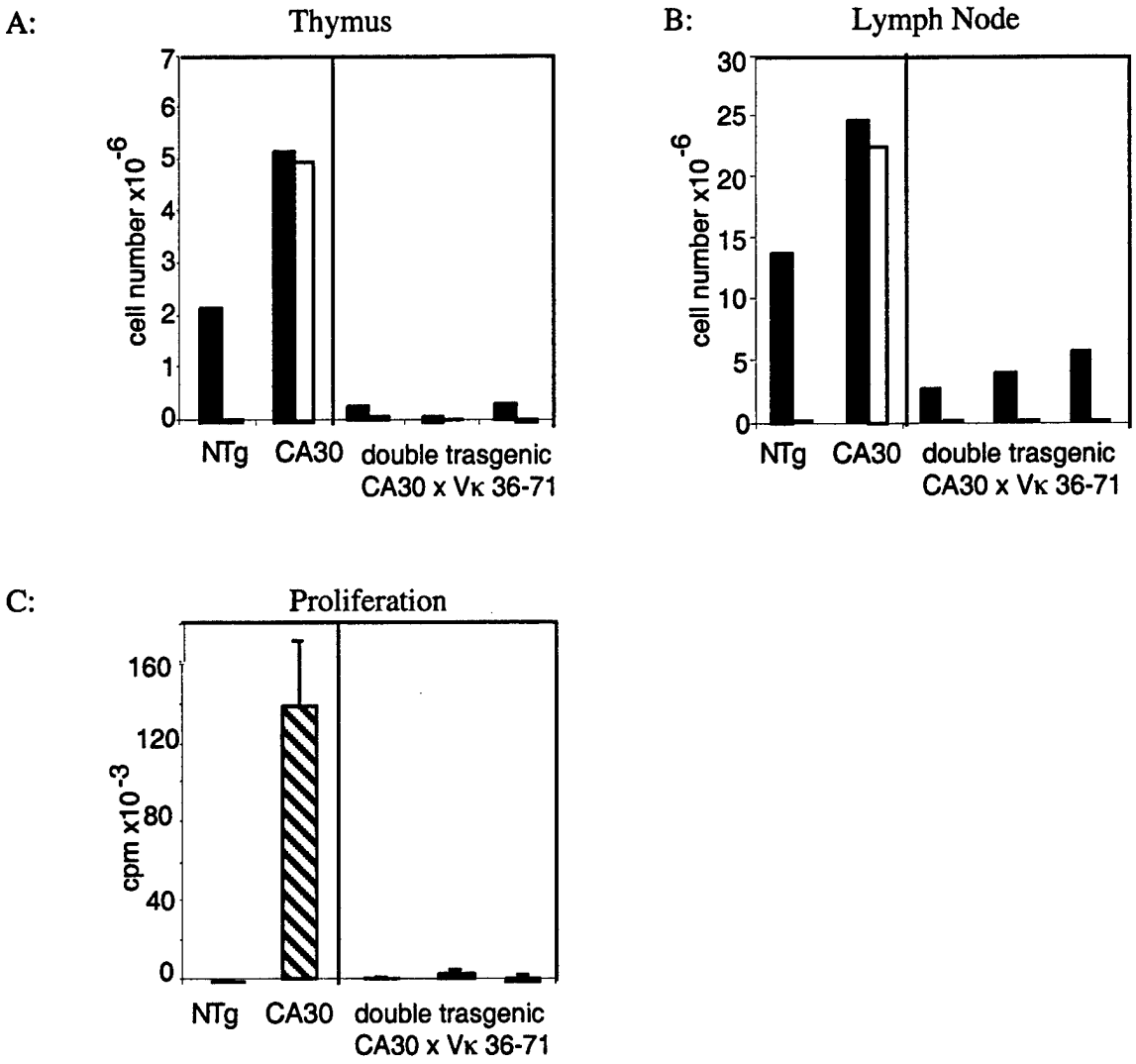


Figure 5: A) Absolute numbers of thymocytes that are CD4 single positive (black bars) and numbers of CD4 single positive cells that are tetramer positive (white bars) in adult CA30 x Vκ 36-71 double transgenic mice, CA30 single transgenic mice (derived from TCR only breeders), or Vκ 36-71 single transgenic mice. B) Cell numbers of CD4 single positive and numbers of CD4 single positive cells that are tetramer positive lymph node cells from the same mice shown in Figure A. C) T cells purified from lymph nodes of mice shown in A and B were stimulated with Vκ 36-71 FR-1 peptide in vitro for 5 days. Thymidine was added for the final 20 hours of culture.

deletion in the thymus. Moreover, soluble antibody alone is capable of inducing Ig-specific T cell tolerance in the thymus. These observations correlated nicely with those of our previous experiments, involving T cell hybridoma production. Despite the level of resolution thus obtained however, it is still unclear whether the a physiological number of Ig-specific T cells will be deleted in the presence of a physiological concentration of their cognate antibody. Thus, ongoing and future experiments involving the production of bone marrow chimeras promise to lead to fundamental observations regarding the nature of T cell responses to immunoglobulin diversity.

Although my analyses confirmed that T cells are tolerant of germline encoded antibody sequences, it remains unclear whether T cells can attain a state of tolerance against rare peptides created somatically through gene recombination. These junctional peptides may often be so rare as to only exist on a handful of B cells per animal. Thus, in Aim #2 of my proposal, I asked whether T cells were tolerant of junctional Ig-diversity and

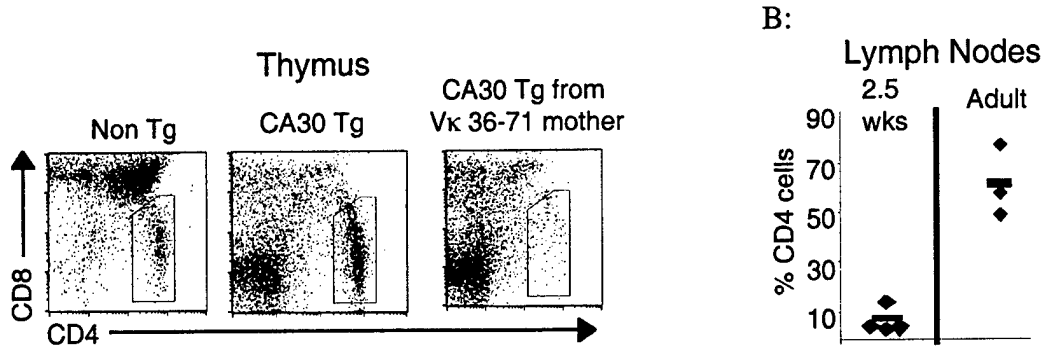


Figure 6: A) Representative FACS plots of thymocytes from 2.5 week-old non-transgenic mice, CA30 transgenic mice and CA30 transgenic mice born of Vκ 36-71 mothers. B) Frequencies of CD4+ cells and among CA30 single transgenic mice born of Vκ 36-71 mothers.

proposed to address this question by immunizing mice with unmutated monoclonal antibodies and testing for T cell responses directed against the third complementarity determining regions (CDR3) of the respective heavy and light chains. We have found 7 interesting T cell hybridomas that were derived from a mouse that was immunized with a monoclonal antibody. An analysis of these hybridomas revealed that they all respond to a pair of synthetic peptides spanning the portion of the heavy chain CDR3 formed at the junctional boundary of the variable (V) and diversity (D) gene segments. Thus, these hybridomas provided me with support for the hypothesis put forth in aim #2 of my proposal. However, as mentioned above, hybridoma sampling is an inefficient method to address the nature of T cell tolerance to multiple Ig-junctional peptides. This along with technical difficulties in developing a consistent and sensitive lymph node proliferation assay necessitated the use of an alternative method in which I simulated an interaction between T and B cells in mediated by a rare somatically-generated Ig V region peptide. To this end, I first adoptively transferred a large number (10^7) CA30 lymph node cells into Vκ 36-71 mice and analyzed the effect on both donor T and recipient B cell populations. As shown in Figure 7, this transfer protocol resulted in massive T cell expansion and B cell activation leading to IgG secretion, some of which was autoreactive. Thus, Ig-directed T-B interactions in the periphery are potentially dangerous. This result indirectly suggests that Ig-specific T cells develop a state of tolerance against Ig V region peptides that are somatically generated because rampant autoreactivity is not a normal biological state.

Because junctional region peptides are often so rare, T cells may remain ignorant to a particular sequence until after B cell activation and antibody secretion. This is especially true given my other findings indicating that resting B cells fail to display Ig-derived peptides (not shown). Thus, to further address the result of Ig-directed T-B interactions in the periphery, I developed a novel adoptive transfer system that recapitulates the expansion of B cells expressing a defined Ig-derived T cell epitope. To this end, I transferred Vκ 36-71 splenocytes into kappa deficient mice (κ-/- mice) as diagramed in Figure 8A. Goat anti-mouse kappa (GαMκ) immunization of the recipients drove the transferred Vκ 36-71 B cells into an immune response that could be detected by secreted antibody and kappa positive germinal centers (Figure 8B and C). In the absence of GαMκ immunization, there were no detectible kappa positive germinal centers or serum kappa antibody (not shown). Thus, I developed a system by which a small number of B cells expressing a defined T cell epitope were participating in an antigen-dependent immune response. By also transferring CA30 T cells into these mice I could closely approximate the activation and expansion of B cells expressing a rare Ig-derived T cell epitope to which the T cell repertoire was not tolerant at the start of the experiment.

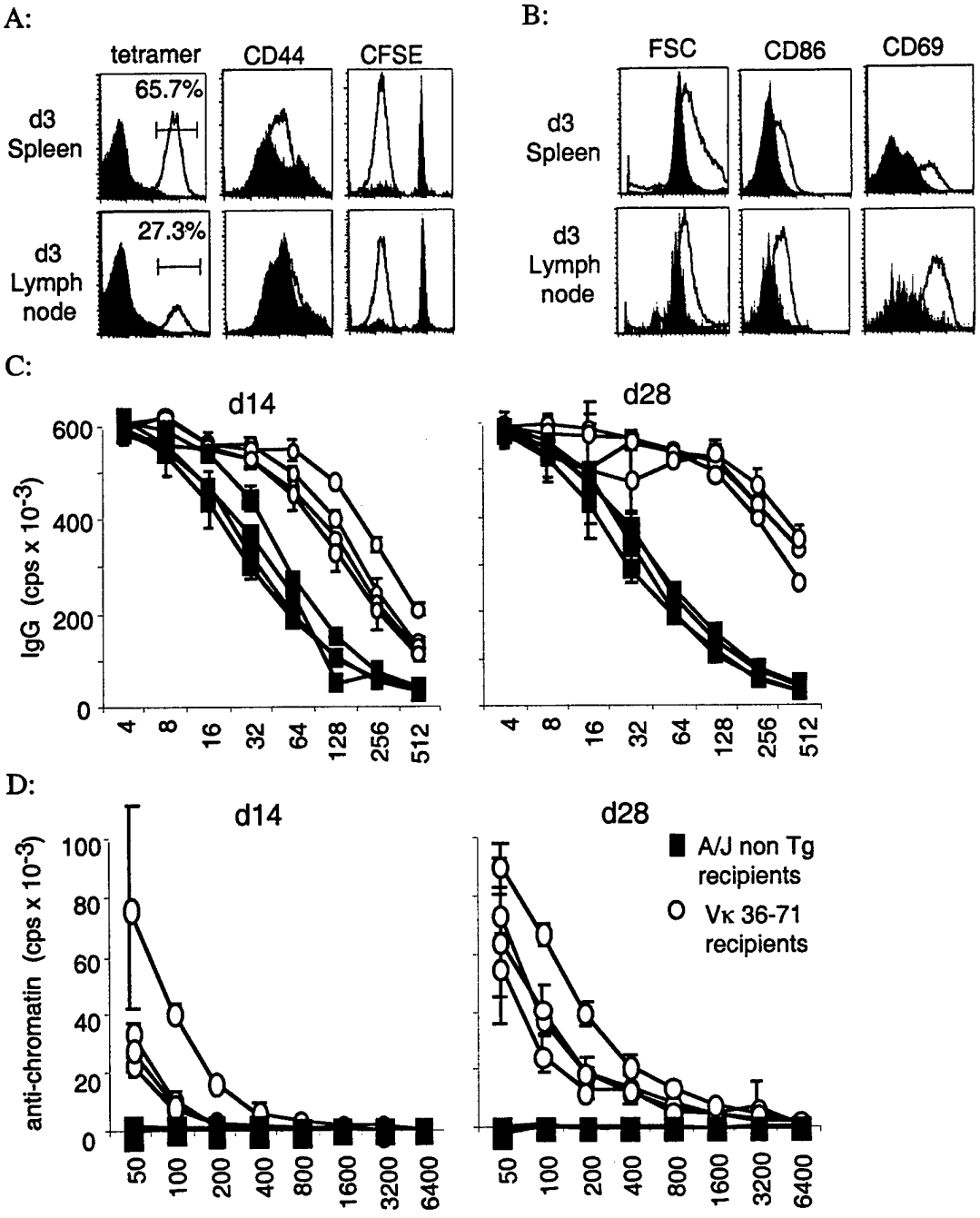


Figure 7: A) FACS analysis of tetramer positive T cells at 3 days after injection of 10^7 CA30 lymph node cells. Tetramer staining is shown for CD4 gated cells. B) FACS analysis of B cells 3 days after CA30 cell transfer. Forward scatter (FSC), CD69 and CD86 expression is shown for B220 gated cells. C) Sera were taken from recipient mice on the indicated days following adoptive transfer of 10^7 CA30 lymph node cells, and tested for total IgG using Goat anti-mouse IgG coated plates. Bound antibody was detected with biotinylated Goat anti-mouse IgG and streptavidin. D) Sera from recipient mice analyzed in C were tested for anti-chromatin IgG antibodies using chromatin-coated plates in a Eu^{+3} based, time-resolved fluoroimmunometric assay. Counts binding to BSA-coated plates were subtracted at each dilution point.

This protocol generated surprising results. First, I found that the level of kappa antibody secreted by Vκ 36-71 B cells was drastically reduced in the presence of CA30 T cells (Figure 9A). Moreover, the kappa positive germinal centers were completely ablated in a V-region specific manner (Figure 9B). It is likely that the reason

these results are different from those found in the first set of experiments (in which 10^7 CA30 cells were transferred into Vκ 36-71 mice) is because the numbers of cells in these experiments are relatively physiological. Strikingly, I also found that T cells that were recovered from these recipient mice were refractory to stimulation *in vitro* indicating that the interaction with activated Vκ 36-71 B cells or soluble Vκ 36-71 bearing antibody ultimately led to a state of T cell tolerance or anergy (not shown). The nature of this refractory phenotype is currently being addressed in our laboratory.

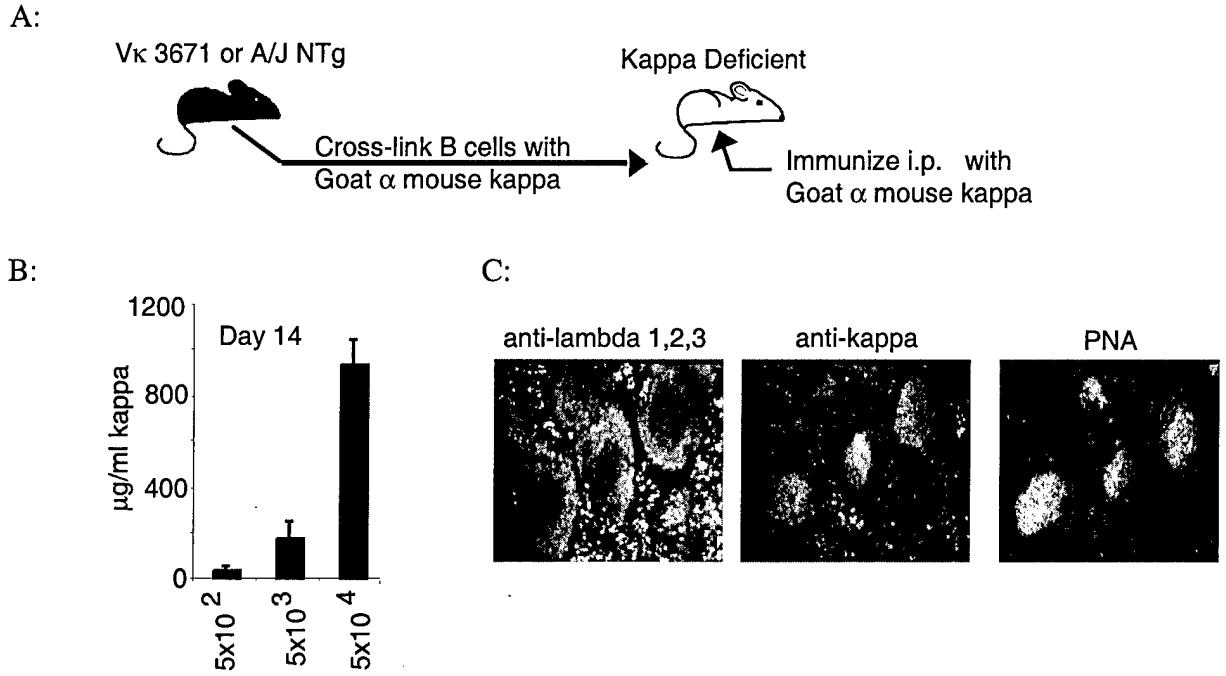


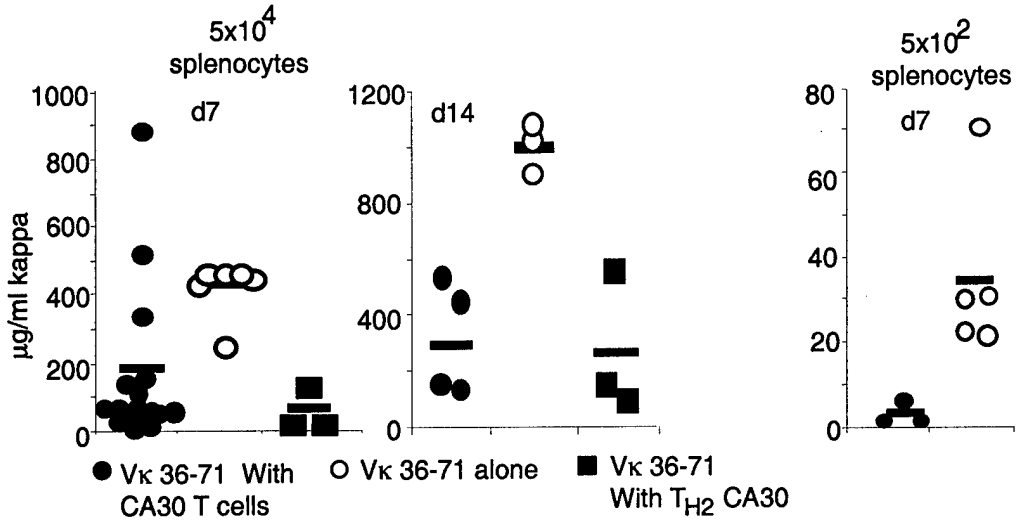
Figure 8: A) Diagram of adoptive transfer protocol. Vκ 36-71 or non-transgenic control splenocytes are cross-linked in vitro with GαMκ and i.v. transferred into kappa deficient (κ-/-) recipient animals. Recipients are immunized with GαMκ immediately following transfer. B) Concentration of kappa antibody in the sera of κ-/- mice 14 days after the transfer of the indicated number of Vκ 36-71 splenocytes as described in Figure A. C) Serial spleen sections of κ-/- mice, 14 days after the transfer of $5x10^4$ Vκ 36-71 B cells were stained with the indicated reagents.

These data also indirectly suggested that T cells attain a state of tolerance to rare Ig-derived diversity. Otherwise, if T cells failed to attain a state of tolerance against Ig-derived sequences, the B cells expressing those sequences would be eliminated during a primary immune response. Because B cell hybridomas have been cloned that contain CDR3-derived T cell epitopes, our evidence indicates that T cells are likely to be tolerant of many junctionally-derived Ig-sequences. Moreover, the Ig-specific T cells seem to be rendered refractory by their interactions with Vκ 36-71 B cells indicating that T cell tolerance and B cell elimination may both result from Ig-directed T-B collaboration in the periphery. The exception to this conclusion is illustrated by the 7 T cell hybridomas mentioned above that respond to CDR3-derived diversity. At first glance, this result seems to be at odds with the idea that T cells are tolerant of junctionally-derived diversity. However, as mentioned, particular junctional peptides are extremely rare. In fact it is likely that some junctional amino acid sequences are not represented in every mouse even among individuals from inbred strains. Thus, it is possible that the mAb used to generate these 7 hybridomas contained a particularly rare junctional sequence.

In a related question, I wanted to understand whether the activated B cells or secreted Vκ 36-71 bearing antibody was causing the CA30 T cell expansion. To this end, I compared the ability of activated Vκ 36-71 B cells and deaggregated mAb 36-71 to stimulate CA30 T cells. As mentioned above, without GαMκ immunization, transferred Vκ 36-71 B cells fail to secrete kappa antibody in κ-/- recipients. As shown in Figure 10, activated Vκ 36-71 B cells (in the absence of secreted antibody) could not stimulate CA30 T cells. In contrast, deaggregated soluble mAb 36-71 was efficient at stimulating CA30 T cells. These experiments

have particular relevance to monoclonal antibody therapy because they show that Ig-specific T cells, if not tolerized, are particularly responsive to deaggregated soluble mAbs. Thus, mAbs containing peptides to which an individual is not tolerant (such as those generated by somatic hypermutation for example), may be particularly immunogenic in recipients (patients), thus leading to a rejection of therapeutic mAb.

A:



B:

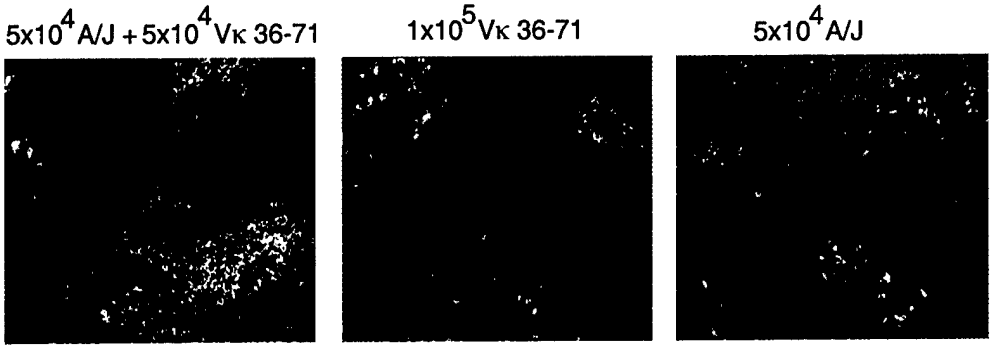


Figure 9: A) Concentration of kappa antibody in sera of κ-/- recipient mice 7 and 14 after the transfer of the indicated number of Vκ 36-71 splenocytes either alone, with $2.5x10^5$ CA30 lymph node cells or with $2.5x10^5$ T_H2 polarized CA30 cells. B) Histological sections were stained with antibodies specific for lambda (red), kappa (green) and PNA (blue). PNA images were taken on a serial section and overlaid in blue with lambda and kappa staining for clarity. The dim green staining seen outside of the germinal centers in mice that received Vκ 36-71 B cells and CA30 T cells (middle column) is the background fluorescence and is observed equally on sections from κ-/- mice that received no kappa positive cells.

Aim #3 of my proposal was an extension of Aim#2 in that it tests more globally the hypothesis that junctional diversity in CDR3 may be a significant source of T cell epitopes in mAbs. The strategy was to make use of mice that either express or lack the enzyme terminal deoxynucleotidyl transferase, or TdT. Mice lacking TdT contain extremely limited junctional diversity within the antibody CDR3. If CDR3 frequently contains immunogenic epitopes for T cells in normal mice, then antibodies from normal (TdT-wild type) mice should be immunogenic with respect to T cells in TdT-deficient mice. This aim is important to the humanization effort, because human antibodies made in mice that carry human transgenes could contain CDR3 epitopes that serve as potential avenues of T cell help for a counter-immune response in human recipients of such mAb. Thus, removing TdT from the animal used to make a mAb may reduce or eliminate a potential counter-immune response in a recipient (patient). In order to address this question, I proposed to study the immunogenicity of mAbs produced in TdT-wild type mice. To this end, I completed the generation of congenic mice on the SWR background that either contain or lack the enzyme TdT. In theory, T cells from these mice should be capable of

responding to antibody CDR3 epitopes created by TdT in wild type mice. Therefore, I have conducted experiments to address the ability of T cells from the TdT-deficient to proliferate in response to epitopes presented by the TdT-wild type strain using the fluorescent dyes CFSE (green) and PKH-26 (red). Fluorescent cell trackers have been used extensively in the literature to follow proliferation in various cell populations [6, 7]. In this case, I labeled T cells with the dyes to track the proliferation of T cells that responded to TdT-derived CDR3 diversity *in vivo*. The results of my experiments revealed no obvious global proliferation within the T cell compartment at the various time points tested. Therefore, it may be rare for a T cell to be specific for CDR3 junctionally diversity, or conversely, it may be very rare for junctional diversity to generate a T cell epitope. However, as discussed in Aim #2, clearly CDR3 derived T cell epitopes are generated. Thus, if these data can be confirmed, they would indicate that the strategy of making anti-tumor mAb in TdT-deficient mice that carry transgenes encoding human immunoglobulin is a worthwhile approach that could lead to benefits in the clinic.

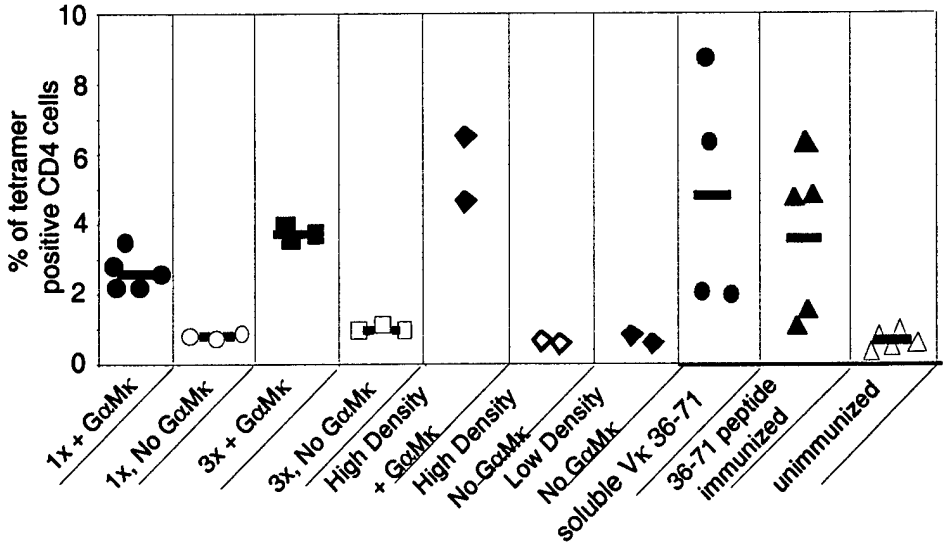


Figure 10: Expansion of tetramer positive cells in individual κ-/- mice. All mice were injected with $2.5x10^5$ CA30 lymph node cells. Recipients were also injected once on day 0 (1x) or 3 times on days 0, 3 and 6 (3x) with Vκ 36-71 splenocytes, injected once on day 0 with $2.5x10^4$ high or low density B cells, injected with 850-1000μg of deaggregated mAb 36-71 (IgG1) or immunized with Vκ 36-71 FR-1 peptide emulsified in CFA. Recipients were either immunized or not with GαMκ as indicated. Tetramer populations are shown as the percent of CD4+, CD16/CD32 negative cells.

Key Research Accomplishments

- Development of a panel of T cell hybridomas from both of the congenic C58 strains of mice and screening of a T cell hybridoma panel generated from the congenic C58 strains. These data support the hypothesis that mice are tolerant of germline encoded antibody diversity.

- Development of the TCR transgenic mice designated CA30 with which high resolution analysis of T cell responses to immunoglobulin-derived epitopes can be observed.

- Development of the I-A^k 36-71 tetrameric staining reagent for use in higher resolution analyses concerning the nature of Ig-specific T cell tolerance.

- Analysis of double transgenic mice expressing both the CA30 T cells and the Vκ 36-71 transgenic B cells. This analysis confirmed our hypothesis that mice are tolerant of germline encoded diversity by revealing the deletion of specific T cells.

- Analysis of CA30 single transgenic mice born of heterozygous Vκ 36-71 mothers. Analysis of these offspring revealed that soluble antibody is sufficient to induce Ig-specific T cell deletion in the thymus.

- Evidence that the heavy chain CDR3 of one mAb contains a T cell epitope.

- Indirect evidence that T cells attain a state of tolerance to Ig-derived sequences since uncontrolled proliferation and autoreactivity are the potential results of Ig-directed T-B collaboration in the absence of T cell tolerance.

- Development of a novel adoptive transfer model system that closely recapitulates the physiological expansion of B cells bearing an Ig-derived T cell epitope.

- Evidence that B cells bearing a defined T cell epitope are eliminated in a V-region specific manner in the presence of V-region specific T cells. This result agrees with our hypothesis that T cells are likely to be tolerant of junctionally encoded epitopes since B cells bearing CDR3-derived T cell epitopes have been cloned.

- Evidence that Ig-specific T cells can be stimulated by deaggregated mAb. This finding agrees with our hypothesis that T cell epitopes present in "humanized" therapeutic mAbs may ultimately induce the counter immune response resulting in mAb rejection.

- Analysis of the proliferative responses of T cells from congenic TdT-deficient animals adoptively transferred into TdT proficient animals. These studies suggested that few T cells from TdT-deficient animals are specific for epitopes generated by junctional diversity.

Reportable Outcomes

- Presentation of an abstract (Keystone Symposia, March 2001)
  Title: "Class II MHC-Restricted Presentation of BCR-Associated Epitopes Dependent Upon B Cell Activation".
  Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki
  Presented by: Christopher M. Snyder

-Presentation of an abstract (Era of Hope, September 2002)
  Title: T Cell Recognition of Monoclonal Antibodies
  Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki
  Presented by: Christopher M. Snyder

- Presentation of a workshop talk (Era of Hope, September 2002)
  Title: T Cell Epitopes in Monoclonal Antibodies: A Risk for Monoclonal Antibody Therapy
  Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki
  Presented by: Christopher M. Snyder

- Presentation of an abstract (Keystone Symposia, January 2003).
  Title: A Novel System for Assessing T Cell Responses to Ig-Variable Region Epitopes Generated by Somatic Hypermutation.
  Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki
  Presented by: Christopher M. Snyder

- Workshop talk (Keystone Symposia, January 2003)
  Title: A Novel System for Assessing the Consequences of T Cell Reactions to Ig V-Regions.
  Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki
  Presented by: Christopher M. Snyder

- Publication of a manuscript in the Journal of Immunology.
  Title: Negligible Class II MHC Presentation of BCR-Derived Peptides by High-Density, Resting B Cells
  Authors: Christopher M. Snyder, Xianghua Zhang, Lawrence J. Wysocki
  Reference: J. Immuol. 2002 168: 3865-3873

- Doctoral Thesis.
  Title: Regulatory Mechanisms Controlling T Cell Cognition of an Ig V-Region Peptide Expressed by Kappa Transgenic B Cells
  Degree: Ph.D.
  Author: Christopher M. Snyder
  Date: 06/09/03

- Manuscript in Preparation:
  Title: Tolerance in CD4+ T Cells Reactive to an Immunoglobulin V Region Sequence
  Authors: Christopher M. Snyder, Ryan A. Heiser, Daniel R. Tonkin, and Lawrence J. Wysocki

- Manuscript in Preparation:
  Title: Idiotype-Expressing B Cells are Eliminated by Idiotype-Specific T Cells During an Immune Response
  Authors: Christopher M. Snyder, Ryan A. Heiser, and Lawrence J. Wysocki

- Production of novel T cell receptor transgenic mouse known as CA30.

- Production of a novel I-A^k tetrameric staining reagent containing the FR-1 peptide from mAb 36-71

Conclusions

The research completed to this point is important in that it allows us to clearly define the observed tolerance of T cells to endogenously encoded immunoglobulin epitopes. Hybridoma sampling has suggested that T cells are tolerant of germline encoded Ig-sequences. Furthermore, analyses of TCR transgenic cells indicate that soluble antibody is sufficient to induce T cell tolerance. The responses of transgenic T cells in the periphery indirectly suggest that T cells are likely to be tolerant of junctionally encoded V-region diversity. Moreover, our results suggest that junctionally encoded T cell epitopes may be rare as assessed by T cell responses in TdT knockout mice. However, our ability to generate 7 CDR3 specific T cell hybrids suggests that junctionally encoded epitopes exist, if at a low frequency. Furthermore, the ability of soluble, deaggregated mAb to induce T cell responses indicates that rare T cell epitopes present in therapeutic mAbs, to which the T cell repertoire is not tolerant, may be highly stimulatory. Therefore it is likely that Ig-specific T cells will be activated and could induce the rejection of the therapeutic mAbs. A further analysis of the T cell responses to the immunoglobulin-derived peptide in various scenarios should further elucidate how T cells might contribute to the counter immune response elicited by monoclonal antibodies. In summary, this research contributes to the expanding body of knowledge relating to the counter-immune response against monoclonal antibody therapies.

References

1. Bogen, B., T. Jorgensen, and K. Hannestad, *T helper cell recognition of idiotopes on lambda 2 light chains of M315 and T952: evidence for dependence on somatic mutations in the third hypervariable region.* Eur J Immunol, 1985. **15**(3): p. 278-81.
2. Eyerman, M.C. and L. Wysocki, *T cell recognition of somatically-generated Ab diversity.* J Immunol, 1994. **152**(4): p. 1569-77.
3. Rees, W., et al., *An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9781-6.
4. Savage, P.A., J.J. Boniface, and M.M. Davis, *A kinetic basis for T cell receptor repertoire selection during an immune response.* Immunity, 1999. **10**(4): p. 485-92.
5. Eyerman, M.C., X. Zhang, and L.J. Wysocki, *T cell recognition and tolerance of antibody diversity.* J Immunol, 1996. **157**(3): p. 1037-46.
6. Bender, J., et al., *CD4+ T cell division in irradiated mice requires peptides distinct from those responsible for thymic selection.* J Exp Med, 1999. **190**(3): p. 367-74.
7. Boutonnat, J., et al., *PKH26 probe in the study of the proliferation of chemoresistant leukemic sublines.* Anticancer Res, 1998. **18**(6A): p. 4243-51. secondary stimulation either in vivo or in vitro.